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<b>(21) International Application Number:</b> PCT/AU97/00467 <b>(22) International Filing Date:</b> 24 July 1997 (24.07.97) <b>(71) Applicant (for all designated States except US):</b> THE AUSTRALIAN NATIONAL UNIVERSITY [AU/AU]; Canberra, ACT 0200 (AU). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BURCH, William, Martin [AU/AU]; 39 Coopernook Avenue, Gympie Bay, NSW 2227 (AU). BROWITT, Rodney, James [AU/AU]; 59 Ashburton Circuit, Kaleen, ACT 2617 (AU). NAIR, Chenicheri, Hariharan [AU/AU]; 22 Flegg Crescent, Gordon, ACT 2906 (AU). SHATS, Elena, Alexandra [AU/AU]; 33 Pelham Close, Chapman, ACT 2611 (AU). <b>(74) Agent:</b> SPRUSON & FERGUSON; G.P.O. Box 3898, Sydney, NSW 2001 (AU).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD FOR DETECTION OF FIBRIN CLOTS  <b>(57) Abstract</b>  The invention relates to a method for labelling macromolecules with a detectable marker which is encompassed in a layer of carbon, and a method for labelling biological macromolecules with such a marker. Disclosed is a method for labelling proteins <i>in vivo</i> with this marker, and a method for detecting fibrin clots using radionuclides contained inside a carbon cage. Preferably the radiolabel is <sup>99m</sup> Tc encapsulated inside the carbon in the form of a nano-encapsulate.		

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## METHOD FOR DETECTION OF FIBRIN CLOTS

## Technical Field

The present invention relates to a method for labelling macromolecules with a detectable marker which is encompassed in a layer of carbon. In particular, it relates to  
5 a method for labelling biological macromolecules with such a marker. More particularly, it relates to a method for labelling proteins *in vivo* with this marker.

Still more particularly, it relates to a method for detecting fibrin clots using radionuclides contained inside a carbon cage, and more particularly, where the radiolabel is <sup>99m</sup>Tc encapsulated inside the carbon in the form of a nano-encapsulate.

10

## Background Art

Currently available methods of labelling macromolecules, and in particular biological macromolecules may be influenced by the chemistry of the detectable marker with which it is desired to label the macromolecule. Ideally, the marker should have no influence on the macromolecule to be labelled. However,  
15 subsidiary effects caused by the chemistry of the marker and a macromolecule being labelled may cause artifactual results.

An application where it is extremely important to gain accurate and specific labelling is the area of clot detection in pathological states such as deep venous thrombosis, thrombophlebitis, and lesions in the vasculature. Morbidity and  
20 mortality caused by intravenous clots is a major public health issue throughout the world. There are several methods of detecting such intravenous clots. For example, use is commonly made of two imaging agents which have similar capabilities for identifying such clots. Fibrinogen labelled with <sup>125</sup>I has been found to be nonspecific and capable of giving false positive results. The second  
25 such radiopharmaceutical is a labelled 3B6 monoclonal antibody.

Technegas is the subject of a patent whose original purpose was to perform high quality diagnostic imaging of airways of the lungs for blood clots. This was primarily used in conjunction with a blood flow agent for the differential diagnosis of blood clots. It is in essence a coating of layers of carbon (varying  
30 between two and ten atoms in thickness) completely enclosing a minute crystal of

<sup>99m</sup>Tc metal, such that it creates a stable inert hydrophobic particle whose overall dimension is between 5 and 30nm in cross-section and about 3nm thick. The production process creates millions of these particles suspended in a carrier gas of argon. In this form they may be inhaled directly and become deposited in the alveolar spaces of the lung. From this distribution, can be created three-dimensional maps of the airways via the gamma ray signal generated by the decay of the Tc from the excited to the ground state (<sup>99m</sup>Tc → <sup>99</sup>Tc).

The possibility of using the particles in liquid suspension as a "nanocolloid" suitable for biological and industrial applications has been investigated. A critical phase of that work was to find the best method of extracting the particles from the argon gas directly into a physiological solution such as saline.

#### Disclosure of the Invention

It has been surprisingly found that nano-encapsulate entities comprising carbon, particularly those of smaller dimensions, bind to macromolecules such as fibrin and soluble fibrin. To do that, in vivo, the nano-encapsulate entities comprising carbon are prepared in an aqueous injectable solution (typically saline or water) and injected intravenously. In vitro the nano-encapsulate entities may be formulated with a suitable carrier and added to a composition containing the macromolecules.

The present invention is based on the ability of carbon nano-phase in the form of nano-encapsulate entities comprising carbon, to bind to macromolecules and simultaneously to be able to enclose a detectable marker. This effectively quarantines the marker from its surrounding environment. The entities are thought to be in the form of layers which in turn form particles.

These entities or particles are distinguished from the standard "Fullerene" derivative particle in that they may contain multiple layers of graphitic carbon; and their method of formation is different. Fullerenes are predominantly produced by a carbon arc process at low pressure, and the residue from the reaction is chemically and physically sorted into separate components for analysis or further processing. The subject nano-encapsulate particles are formed by a resistive or otherwise controlled thermal graphite heating process. The co-condensation reaction in which the

macromolecules are formed seems to occur in the boundary region of a stable thermal plasma that has been termed a "thermo-pause". Time, temperature and pressure conditions for the "thermopause" determine the size of the native metal crystal and the number of graphitic layers which surround it.

5 According to one embodiment of this invention there is provided a reagent for use in labelling a macromolecule, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon. Generally the reagent is capable of binding to the macromolecule.

10 The reagent for use in labelling macromolecules, may comprise a detectable marker contained in shrink wrapped graphite around said marker. Typically a composition is provided comprising the reagent and a suitable carrier, diluent, excipient and/or adjuvant.

15 According to a second embodiment of this invention there is provided a method for labelling a macromolecule with a detectable marker, which method comprises contacting said macromolecule with the reagent of the first embodiment whereby the reagent binds to the macromolecule to form a reagent:macromolecule complex.

The method for labelling a macromolecule with a detectable marker, may comprise contacting said macromolecule with a detectable marker which has been shrink wrapped in graphite surrounding said marker.

20

The method of the second embodiment may further comprise detecting the reagent macromolecule complex by detecting the detectable marker.

25 According to a third embodiment of this invention there is provided a method for labelling a biological macromolecule in a patient, which method comprises administering to the patient, an effective amount of the reagent of the first embodiment.

The method for labelling a biological macromolecule in a patient, may comprise administering to the patient, an effective amount of a composition comprising a detectable marker, inside shrink wrapped graphite surrounding said marker.

30 Suitably, the reagent is administered in the form of an injectable composition containing a suitable carrier (e.g. saline, typically 0.1N - 2.5N saline, more typically 0.5N - 2N saline, and more typically 0.8-1.2N saline and more typically about 1N saline). Typically the composition contains less than 100ng of detectable marker in the carbon.

The macromolecule to be labelled may be any macromolecule amenable to such labelling with carbon. Typically, such a macromolecule would be a biological macromolecule in an *in vivo* or *in situ* setting. A specific example of such a macromolecule is a protein, of which a specific example is fibrin.

5 The detectable marker may be detectable by radiochemical techniques such as a radionuclide, by magnetic resonance imaging or by visual methods. The detectable marker may be pharmaceutically or veterinarily acceptable or otherwise (e.g. laboratory or analytically acceptable) depending on the intended use of the reagent.

Generally, radionuclides which are traditionally used in imaging techniques are suitable for use in this invention. Examples of suitable radionuclides are <sup>111</sup>In with a half-life of 2.8 days, <sup>67</sup>Ga, <sup>68</sup>Ga, <sup>99m</sup>Tc. A preferred radionuclide is <sup>99m</sup>Tc.

An example of a detectable marker suitably for use in magnetic resonance imaging techniques is Gd.

15 Examples of markers detectable by visual techniques are colloidal compounds which exhibit a distinctive colour. An example is colloidal gold.

The detectable marker is preferably in the form of or enclosed in a nano-encapsulate carbon entity or particle. In particular, it should be of a thickness such that the chemistry of the detectable marker is not manifested in the environment outside of the nano-encapsulate comprising carbon. Preferably, the carbon is 2 to 10 layers thick.

20 According to another embodiment of this invention there is provided a method of forming reagent for use in labelling a macromolecule, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon comprising the steps of:

- 25 (a) depositing a solid form of the detectable marker onto a carbon crucible; and  
(b) heating the carbon crucible in a sealed container to a temperature in a range so as to form the reagent for use in labelling a macromolecule, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon, said reagent being capable of binding to the macromolecule.

30 Generally the method further comprises precipitating the reagent.

According to another embodiment of this invention there is provided a method of forming reagent for use in labelling a macromolecule, comprising a detectable

marker encapsulated by a nano-encapsulate entity comprising carbon comprising the steps of:

depositing a liquid solution of a detectable marker onto a carbon crucible;

evaporating the liquid so a solid residue of the detectable marker remains in the crucible; and

heating the carbon crucible in a sealed container to a temperature in a range so as to form the reagent for use in labelling a macromolecule, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon, said reagent being capable of binding to the macromolecule.

Generally the method further comprises precipitating the reagent.

Where the detectable label is  $^{99m}\text{Tc}$  the liquid solution of technetium is a liquid solution of sodium pertechnetate.

The invention includes reagents when made by the above processes.

Generally the crucible is heated to a temperature in the range 2250 - 2900°C more typically 2470-2900°C, more typically about 2700°C. Typically the crucible is pulse heated at from 0.1 - 5 secs, more typically 1 - 3 seconds, more typically 2.7 secs. Typically the carbon crucible is a graphite crucible, more typically a pure graphite crucible. The graphite may be pyrolytic graphite or porous standard graphite, for example. Pyrolytic graphite is preferred. Generally the graphite crucible is heated in the presence of a substantially pure inert gas atmosphere within the sealed container. Typically the inert gas is argon. The crucible may be heated in the presence of oxygen within the sealed container. Typically the oxygen concentration within the sealed chamber is 5% or less, more typically 3% v/v or less and the remainder of the gaseous atmosphere is an inert gas such as argon, typically pure argon. Where the detectable label is  $^{99m}\text{Tc}$  the solid form of technetium is typically sodium pertechnetate.

Suitable methods for preparing detectable markers such as radionuclides, encapsulated in a nano-encapsulate entity comprising carbon are generally described in Australian Patent No. 589 578 (incorporated herein by cross reference). However, unlike heating the graphite crucible containing sodium pertechnetate to any temperature above at least 1900 or 2200°C under an inert atmosphere so as to produce Technegas as described in Australian Patent No. 589 578 (traces of oxygen of 0.1-3% v/v may be included to produce varying concentrations of Pertechnegas) it is important to only heat the graphite crucible to a temperature in the range 2250 - 3000°C, more typically 2475

- 2950°C, more typically 2470 - 2900°C, and even more typically about 2700°C. Generally pulse heating (0.5 - 3 secs) is used, in order to produce nano-encapsulates comprising carbon that will bind to a macromolecule such as, for example, fibrin. Heating the graphite crucible to a temperature within a different temperature range may  
5 be required for binding of the resultant nano-encapsulate to a different macromolecule but this temperature range can be readily determined by simple trial and error. In addition, any detectable marker that can be incorporated under the conditions of preparation can be substituted for <sup>99m</sup>Tc described in AU 589 578. Alternatively, a detectable marker may be left out and the nano-encapsulate comprising carbon thus  
10 produced is then precipitated (using an electrostatic precipitator of the type described in AU 31778/95 (incorporated herein by cross reference), for example, and the resultant precipitate is then formulated with a desired carrier, or adjuvant, for example at a desired concentration (which will be dependent on the end use).

The electrostatic precipitator may include a housing defining a duct through which  
15 a gas containing carbon particles passes, the duct having an inlet and an outlet; an ion source past which the gas passes to charge the particles; an electrode between the inlet and the outlet and spaced downstream from the ion source; means to establish an electric potential between the ion source and the electrode; and wherein the electrode is coated with a soluble material to which the particles are attracted so as to become  
20 deposited thereon. An electrode for the electrostatic precipitator, may include a coating of a soluble material upon which the particles are deposited by being attracted thereto. An alternative electrostatic precipitator to collect particles from a gas stream, may include a duct through which the gas passes between an inlet and an outlet; an ion source between the inlet and the outlet and past which the gas passes to have the particles  
25 charged; a reservoir containing a liquid past which the gas passes; and means to establish an electric potential between the ion source and the reservoir so that particles are attracted to the liquid. Another alternative electrostatic precipitator, may include a housing defining a duct through which a gas containing carbon particles passes, the duct having an inlet and an outlet; an ion source projecting into the duct and located between  
30 the inlet and the outlet; a wall surrounding at least part of the duct between the inlet and the outlet; means in the duct to receive a liquid; means to enable the establishment of an electric potential between the ion source and the liquid; means to cause the liquid to produce droplets to be dispersed in the duct; and wherein upon the application of the



electric potential, the droplets and particles are attracted to the wall. A method of collecting nano-encapsulate entities/particles comprising carbon may include the steps of passing a gas stream containing the entities/particles, through a chamber, the gas stream including an inert gas and air; passing the gas stream past an ion source within  
5 the chamber to charge the particles; attracting the particles to an electrode by establishing an electrical potential between the ion source and the electrode. The entites/particles are then removed from the electrode and formulated as required.

The invention includes a reagent for use in labelling macromolecules, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon when  
10 made by the above process.

In a particular application of this invention, the radionuclide is  $^{99m}\text{Tc}$  encapsulated in 2 to 20 layers more typically 2 to 10 layers of carbon and is used in detecting the presence of a fibrin clot in the subject.

Thus, according to a fourth embodiment of this invention there is provided a  
15 method for detecting and determining the location of a fibrin clot in a patient, which method comprises administering to the patient a pharmaceutical composition comprising a reagent of the first embodiment and a pharmaceutically acceptable carrier and detecting any reagent:fibrin complex formed in vivo in the patient.

The method for detecting and determining the location of a fibrin clot in a patient,  
20 may comprise administering to the patient a composition comprising a detectable marker contained in shrink wrapped graphite around said marker in a suitable medium.

It has been found that addition of a trace of surfactant coating to the particles enhances their binding efficiency by up to 10 times. Suitably, the surfactant is  $\text{C}_{16}\text{EO}_6$ . The concentration of this surfactant which enhances the binding efficiency is  
25 suitably between 0.001% and 0.010% v/v more typically, 0.003% and 0.006% v/v. A further surfactant which is suitable is cyclodextrin.

Thus, according to a fifth embodiment of this invention, there is provided a method for detecting the presence of fibrin in a composition, which method comprises contacting the composition with a reagent of the first embodiment and determining  
30 whether any fibrin:reagent complex is present wherein presence of a fibrin reagent complex is indicative that fibrin is present in the composition and absence of fibrin:reagent complex is indicative that fibrin is not present in the composition.

The fifth embodiment may include the step of separating any fibrin:reagent complex from the composition prior to detecting for its presence.

The applicants have also found that nano-encapsulate entities comprising carbon by themselves, i.e. carbon nano-encapsulate particles by themselves (i.e. those which  
5 do not include via encapsulation another entity such as a detectable label), may bind to macromolecules (e.g. proteins such as fibrin).

Thus, according to a sixth embodiment of this invention, there is provided a method for detecting the presence of a macromolecule in a source, which method comprises contacting the source with a reagent for use in labelling macromolecules,  
10 comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon, the reagent being capable of forming a complex with the macromolecule if present, and detecting for the presence or otherwise of a macromolecule:reagent complex wherein presence of a macromolecule:reagent complex is indicative that the macromolecule is present in the source and absence of macromolecule:reagent complex  
15 is indicative that the macromolecule is not present in the source.

The fifth and sixth embodiments of this invention may be suitably employed in techniques such as dip-stick methods, test strip methods, ELISA methods, agglutination tests using latex suspensions, and the like. These methods may make use of antibodies bound to the carbon nano-particle, for example. Alternatively, in the case of latex, dip-  
20 stick methods and test strip methods a macromolecule binding molecule may be attached to the latex, dip stick or test strip (or other suitable substrate e.g. beads such as glass beads). Thus when such a latex particle, dip stick or test strip is dipped into or placed in contact with a composition containing macromolecule:reagent complex it will bind the macromolecule portion of the complex (as well as any free macromolecules or  
25 other macromolecule complexes in the composition). The latex particle, dip stick or test strip is then typically washed so as to remove any unattached components of the composition and whether and/or the amount of complex attached to the latex particle, dip stick or test strip determined by detecting whether the detectable label is present (and, if so, the amount present if required) on the latex particle, dip stick or test strip.  
30 Typically the macromolecule binding molecule is an antibody to the macromolecule (e.g. antifibrin in the case where the macromolecule is fibrin).

The methods of the invention are not restricted to human patients. Animal experiments have proven that this binding to Fibrin occurs in deposits in Deep Vein

Thrombosis, Pulmonary Embolism and malignant Disease (Skin Cancer). The methods of the invention can be practised on mammals including a bovine, human, ovine, equine, caprine, leporine, feline or canine vertebrate. Most often the invention will be practised on a human patient. The composition would then be a pharmaceutical composition and the carrier, diluent, excipient and/or adjuvant would be pharmaceutically acceptable. Where the mammal is not human, the composition would be typically a veterinary composition and the carrier, diluent, excipient and/or adjuvant would be veterinarily acceptable. However, the methods of the fifth and sixth embodiments of the invention may be performed either in vivo or in vitro (including a reaction in a suitable laboratory apparatus such as a test tube or beaker followed by separation of any complex formed and detection for the presence of complex).

In this specification and claims the expression "nano-encapsulate" implies that the nano-encapsulate entity comprising carbon is at least capable of a encapsulating or enclosing, typically tightly enclosing or encapsulating a detectable marker by the carbon. An explanation for "encapsulation" is that the phenomenon may be the result of co-condensation. That is, the detectable marker crystals or liquid droplets or amorphous deposits first form and then carbon condenses around that marker. The carbon forms a nano-encapsulate entity so as to encapsulate the marker to form the reagent of the first embodiment. Where a marker or metal is not required to be encapsulated carbon is simply prepared so as to form a nano-encapsulate entity comprising carbon.

Advantageously the patient is a mammal or vertebrate and is a bovine, human, ovine, equine, caprine, Leporine, feline or canine vertebrate. Advantageously the vertebrate is a bovine, human, ovine, equine, caprine, Leporine, domestic fowl, feline or canine vertebrate.

Typically the reagent is formulated as a pharmaceutically acceptable composition or a veterinarily acceptable composition depending on its intended use.

More typically, the mammal is a human and the composition is a pharmaceutically acceptable composition which includes at least one pharmaceutically acceptable carrier, adjuvant and/or excipient.

Where, the mammal is an animal the composition is generally veterinarily acceptable composition which includes at least one veterinarily acceptable carrier, adjuvant and/or excipient.

For parenteral administration, the reagent of the first embodiment may be prepared in sterile aqueous or oleaginous solution or suspension. Suitable non-toxic parenterally acceptable diluents or solvents include water, Ringer's solution, isotonic salt solution, 1,3-butanediol, ethanol, propylene glycol or polyethylene glycols in mixtures with water.

5 Aqueous solutions or suspensions may further comprise one or more buffering agents. Suitable buffering agents include sodium acetate, sodium citrate, sodium borate or sodium tartrate, for example.

The dosage form of the reagent composition will comprise typically from 0.001% to 10% by weight of the reagent of the first embodiment. Usually, dosage forms according to the invention will comprise from 0.1% to about 5% by weight of the reagent of the first embodiment.

10

Compositions of the invention may be prepared by means known in the art for the preparation of compositions (such as in the art of preparing veterinary and pharmaceutical compositions) including blending, grinding, homogenising, suspending, dissolving, emulsifying, dispersing and mixing of the reagent of the first embodiment together with the appropriate excipient(s), carrier(s), adjuvant(s) and/or diluent(s).

15

In methods of the invention, the reagent compositions may be administered orally or parenterally, e.g. by injection and by intra-arterial infusion, rectally or by inhalation spray.

20 A suitable method may comprise the administration of a single dose or multiple doses. If more than one type of reagent is involved in a method of the invention (e.g. there may be multiple reagents with different detectable markers) the reagents can be administered at the same time or at different times (including sequentially).

The administered dosage of the reagent(s) can vary and depends on several factors, such as the condition, age and size of the patient as well as the nature of the macromolecule and the effectiveness of the binding of the reagent to form the macromolecule:reagent complex. Dosages or reagent will typically range from 0.0001mg to 200 mg per kg. Usually, the dose of the reagent will be from 0.001mg to 10mg per kg of body weight.

25

For oral administration, the pharmaceutical or veterinary composition may be in the form of tablets, lozenges, pills, troches, capsules, elixirs, powders, including lyophilised powders, solutions, granules, suspensions, emulsions, syrups and tinctures. Slow-release, or delayed-release, forms may also be prepared, for example in the form of coated particles, multi-layer tablets or microgranules.

30

For parenteral administration, the reagent(s) may be prepared in sterile aqueous or oleaginous solution or suspension. Suitable non-toxic parenterally acceptable diluents or solvents include water, Ringer's solution, isotonic salt solution, 5% dextrose in water, buffered sodium or ammonium acetate solution, 1,3-butanediol, ethanol, propylene glycol or polyethylene glycols in mixtures with water. Aqueous solutions or suspensions may further comprise one or more buffering agents. Suitable buffering agents include sodium acetate, sodium citrate, sodium borate or sodium tartrate, for example. Aqueous solutions for parenteral administration are also suitable for administration orally or by inhalation.

Suitably, an inhalation spray comprising an reagent(s) of the first embodiment will be in the form of a solution, suspension or emulsion as exemplified above. The inhalation spray composition may further comprise an inhalable propellant of low toxicity. Suitable propellants include carbon dioxide or nitrous oxide.

Compositions of the invention may be prepared by means known in the art for the preparation of pharmaceutical compositions including blending, grinding, homogenising, suspending, dissolving, emulsifying, dispersing and mixing of the compound of formula (I) together with the selected excipient(s), carrier(s), adjuvant(s) and/or diluent(s).

Examples of dosage forms in accordance with the invention are as follows:

1. Injectable solution

	Reagent(s) of the first embodiment	0.01 to 20 mg, generally 0.1 to 10mg
20	Sodium chloride	8.5 mg
	Potassium chloride	3 mg
	Calcium chloride	4.8 mg
	Water for injection, q.s. to	10 ml

Illustrative of typically used pharmaceutically or veterinarily acceptable carriers or diluents are demineralized or distilled water; isotonic saline solution. Typically, the carrier or carriers will form from 90% to 99.9% by weight of the composition.

Suitable buffering agents include salts of boric, acetic, phosphoric, citric, malic, succinic acids and the like, for example sodium citrate, sodium bicarbonate, sodium acetate and sodium phosphate. Additionally or alternatively, the free acids may be used, together with an alkali such as sodium hydroxide, sodium carbonate, sodium bicarbonate, potassium hydroxide, potassium carbonate or potassium bicarbonate. Typically, the buffering agent or agents will form from 0.1% to 20% by weight of the composition.

The following expressions are used in in the Examples.

- a) **Fullertag**: (Registered Trademark) A nanocolloid suspension (in saline or distilled water) of nano-encapsulates which are 2-10 atomic layers of carbon enclosing technetium crystal such that it forms a stable inert hydrophobic or hydrophylic particle whose overall dimension is between about 5 and about 30nm in cross-section and about 3nm thick. b) **ThromboTrace**: *Fullertag* with surfactant. In the Examples, the surfactant is C<sub>16</sub>EO<sub>6</sub>.

### Brief Description of the Drawings

Figure 1 is an autoradiograph of a fibrin clot in accordance with Example 12;

- 10 Figure 2 is a schematic representation of the artificial circuit described in Example 13;

Figure 3 shows the accumulation of radioactivity in the intramural clot in the artificial circuit of Example 13;

Figure 4 shows the transmission electron micrography described in Example 14;

- 15 Figure 5 shows imaging of clots in a rabbit ear vein described in Example 17;

Figure 6 shows a clot caused by intramuscular injection of anaesthetic described in Example 18;

Figure 7 shows the imaging of a clot in a cat femoral vein described in Example 19;

- 20 Figure 8 shows the imaging of a clot in rabbit pulmonary vasculature described in Example 20;

Figure 9 shows the autoradiography of rabbit lungs with pulmonary emboli also described in Example 21;

- 25 Figure 10 shows the labelling of a small tumour in the ear of the rabbit as described in Example 23; and

Figure 10 shows nano-encapsulates with colloidal gold.

### Best Modes and Other Modes For Carrying the Invention

It is envisaged that the binding of nano-encapsulates can be used for:

- 30 a) imaging of clots in blood vessels in patients with deep vein thrombosis (DVT), patients with atheromatous occlusions in the vasculature and embolisms;
- b) the development of target specific drugs for the dissolution of blood clots;

- c) the use of technology to develop a bedside measurement of fibrin(ogen) soluble fibrin;
- d) the detection of early tumours; and
- e) research tools to study clots and diseases like atherosclerosis.

5 The mechanism for formation of Carbon nano-encapsulates depends on a co-condensation of the native metal crystals with the C<sub>3</sub> gas that is present above carbon at high temperatures. The reaction is carried out in an inert gas atmosphere, typically argon. Thus, the final nano-encapsulated sized of the particles can be modified by varying the rate and duration of the heating cycle. At present, repeat short pulses of

10 heat for 2.7sec to temperatures as high as 2700°C will shrink-wrap particles that go to the lower limit of resolution of electron microscopes namely 5nm. Pyrolytic or glassy-carbon provides a better yield of activity of Tc from the crucible than the more porous standard graphite. This appears to be due to the non-absorption of the ions into the graphite matrix. Four lots of 2.7sec heating pulses to 2700°C in pyrolytic or glassy-

15 carbon gives rise to a much smaller spectrum of particle size distribution than the original heating cycle of 2500°C for 15sec. The carbon nano-encapsulates were precipitated on an electrode using a precipitator as described in Australian Patent Application No. 31778/95 and formulated in saline. The reagent composition may be then injected into a patient and detected in vivo so as locate fibrin deposits.

20 Typically the starting material is Tc-99m as standard sodium pertechnetate for injection. The Micro-Aerosol Generator (MAGen - USA name ), or carbob nano-encapsulate generator is essentially a miniature high temperature furnace in which the heating element is also the source of graphite and/or carbon vapour which ultimately coats the Technetium metal. The heating element is made from 100% pure spectroscopic graphite

25 whose electrical and mechanical specifications match the requirments of the machine. It is a 6mm square section rod 50mm long, machined to form a crucible in the centre section such that it can hold a liquid volume of 0.14mL. This hollowed and thinned section also provides the high resistive portion of the rod which becomes the hottest section when electric current is passed through it. The rod is held under spring tension

30 between two high current electrodes. The entire assembly is mounted as a drawer section sliding into the lower chamber of a 6L vessel, and electrically powered from an automatic process-controller. The crucible is first filled with liquid Sodium Pertechnetate in normal physiological saline, which in most instances contains enough

activity [ 260-370MBq or 7-10mCi ] for a single patient administration. The drawer section is then closed and the automatic process takes over, gently blowing pure argon gas over the top of the crucible while warming it to 70°C. This is known as the "simmer" cycle, and takes 6 minutes, during which time the liquid in the crucible dries out and the whole chamber is purged with pure argon, replacing all the original air and water vapour. A process controller alerts the user that the machine is ready to produce carbon nano-encapsulates. Traces of oxygen as low as 0.1% may be included up to 3% v/v. At the conclusion of this preparation phase, the machine is activated via the control panel "start" button, and the crucible temperature rises to 2550°C by resistively heating it with about 4.5kW of power within 0.75s (four lots of 2.7sec heating pulses to 2700°C in pyrolytic or glassy-carbon gives rise to a much smaller spectrum of particle size distribution than the original heating cycle of 2550°C for 15sec) and holds that value within 50°C for 15s through a feedback servo from an optical sensor before switching off. This fills the 6L chamber with gas comprising carbon nano-encapsulates which encapsulate Tc-99m. The nano-encapsulate entities/particles are collected by passing the gas stream containing the entities/particles, through a chamber, the gas stream including an inert gas and air; passing the gas stream past an ion source within the chamber to charge the particles; attracting the particles to an electrode by establishing an electrical potential between the ion source and the electrode. The entities/particles are then removed from the electrode and formulated with a carrier as required. The present invention will now be described with reference to the following examples which should not be construed as limiting on the scope thereof.

#### Example 1

Carbon nano-encapsulates were prepared in accordance with the methods of Australian Patent No. 589 578 except the graphite crucible was subjected to four lots of 2.7sec heating pulses to 2700°C in pyrolytic or glassy-carbon, in order to produce nano-encapsulates comprising carbon that will bind to a macromolecule such as, for example, fibrin, the disclosure of Australian Patent No. 589 578 is hereby incorporated by reference.



**Example 2**

Carbon nano-encapsulates were formulated into normal saline by precipitating the carbon nano-encapsulates formed as per Example 1 using precipitation apparatus and methods of Australian Patent Application No. 31778/95, the disclosure of which is hereby incorporated by reference and then mixing the desired amount of the carbon nano-encapsulates with normal saline.

**Example 3**

Plasma was mixed with a small aliquot of nano-encapsulates formulated into saline in accordance with Example 2 and clotted with 1U/mL thrombin. After incubation at room temperature for 1 hour the plasma clot was crushed using a wooden spatula. A gamma counter was used to count radioactivity. Radioactivity was confined to the fibrin with less than 3% of the original activity resident in the exudate.

**Example 4**

A similar experiment using purified fibrinogen was performed to exclude binding to other plasma proteins. Again it was shown that only about 3% of the radioactivity was resident in the exudate.

Both these experiments show that these nano-encapsulates have specific binding capacity for fibrin.

**Example 5**

To ensure that such labelling could not be transferred or leached from clots to whole blood or plasma, plasma clots were incubated in whole blood or plasma at 37°C for 3 hours. The radioactivity was confined to the clot with very little escaping to the surrounding blood or plasma (0.7% radioactivity in blood and plasma, respectively).

**Example 6**

Unlabelled plasma clots and clots developed from purified fibrinogen solution in glass tubes respectively were gently permeated with nano-encapsulates formulated into saline in accordance with Example 2. The initial washout of serum was devoid of radioactivity as expected. Further measurement showed that most of the nano-encapsulates were specifically bound to fibrin. These clots were removed from the

glass tubes and crushed and carefully washed three times in saline and counted in a gamma-counter. Radioactivity was confined to the clot and barely detectable in the exudate.

#### Example 7

5 Tests of the efficacy of the fibrin label were performed *in vivo*, using the rabbit, which is the standard model for vascular research. This experiment was performed on multiple occasions on different animals. One ear was given a small needle stick injury and nano-encapsulates formulated into saline in accordance with Example 2 in 1mL volume was injected in the other ear. The rabbit was anaesthetised for the whole  
10 procedure and laid on the detector surface of a gamma camera (in this instance a GE model 400T). In the second animal there was a further small vascular injury and bruise in the ear on the side of the injection.

The injury on the ear opposite to the injection site showed an increased uptake on the "first pass" of the blood flow through the body. In the second rabbit, the injury  
15 proximal to the injection site labelled much more strongly than that in the other ear, but then washed out over a period of twenty minutes. Dynamic analysis showed that the initial uptake and the wash-out followed by a renewed uptake slowly with time, occurred in both animals at about the same rate.

Examples 8-16 and 22-25 relate to *in vitro* studies while Examples 17-21 relate to  
20 *in vivo* studies.

#### Example 8

##### Binding of *ThromboTrace* to fibrin.

Protocol: Fibrin network developed from 1 mL pooled human plasma obtained from healthy donors (by adding 1U/mL bovine thrombin(Parke Davis) and  
25 25mM $\text{CaCl}_2$  (final concentration)) was incubated with normal saline (NS) (4mL) containing *ThromboTrace* (TT) (100 $\mu\text{L}$ ) at 37°C for 2 hours using an elliptical rotator. A mixture of normal saline and *ThromboTrace* without clot was used as control. Clots were then crushed with a wooden spatulum and counted using a gamma counter. Nanocolloid preparation of  $^{99}\text{Tc-Sb}_2\text{S}_3$  (antimony sulphide) used for bone imaging was  
30 studied as a control.

##### Results:

50 $\mu$ l NS + TT (initial count)	99500cpm	50 $\mu$ l NS + $^{99}\text{Tc-Sb}_2\text{S}_3$ (initial count)	397800cpm
50 $\mu$ l NS + TT (post-incubation)	87450cpm	50 $\mu$ l NS + $^{99}\text{Tc-Sb}_2\text{S}_3$ (post-incubation)	415000cpm
50 $\mu$ l NS + TT + clot (post-incubation)	72870cpm	50 $\mu$ l NS + $^{99}\text{Tc-Sb}_2\text{S}_3$ + clot (post-incubation)	408700cpm
Crushed clot on spatulum After extensive wash in NS	478720cpm 457436cpm	Crushed clot on spatulum after 3 washes in NS after 6 washes in NS	9430cpm 7790 3150

Conclusion: There is a significant decrease of the label (17%) in the incubation medium with *ThromboTrace* which accounts for the high count observed in the crushed fibrin clot. There is no significant changes in the activity of incubation medium with nanocolloid and there was no significant binding of  $^{99}\text{Tc-Sb}_2\text{S}_3$  to fibrin, after 6 washes most of the activity washes away. This observation suggests a high specificity of *ThromboTrace* for fibrin.

#### Example 9

Investigation of the strength of *ThromboTrace* binding to fibrin.

Protocol: *ThromboTrace* (TT) (50 $\mu$ l) was added to pooled plasma obtained from the blood of healthy donors (5mL). Fibrin networks were developed from 1 mL of this labelled plasma (by adding 1U/mL bovine thrombin (Parke Davis) and 25mM  $\text{CaCl}_2$  (final concentration)) and allowed to incubate at 37°C for 30 min. Clots were then crushed with wooden spatulum, extensively washed with normal saline and counted using a gamma counter. Nanocolloid preparation of  $^{99}\text{Tc-Sb}_2\text{S}_3$  (antimony sulphide) used for bone imaging was studied as a control.

Results:

50 $\mu$ l plasma + <i>TT</i> (initial count)	58700cpm	50 $\mu$ l plasma + $^{99}\text{Tc}$ - $\text{Sb}_2\text{S}_3$ (initial count)	100195cpm
50 $\mu$ l expelled serum (after clot crushed)	1600cpm	50 $\mu$ l expelled serum (after clot crushed)	100500cpm
Crushed clot on spatulum	1026700cpm	Crushed clot on spatulum	3650cpm
Crushed clot on spatulum  after extensive wash in  normal saline	1005850cpm	Crushed clot on spatulum  after extensive wash in  normal saline	1800cpm

**Conclusion:** Fibrin clot retained 97% of the initial radioactivity of *TT* solution which was tightly bound. There was no binding of nanocolloid  $^{99}\text{Tc}$ - $\text{Sb}_2\text{S}_3$  to fibrin.

#### Example 10

Study of the washout of *ThromboTrace* from clot.

- 5      **Protocol:** *ThromboTrace* (*TT*) (50 $\mu$ l) was added to pooled plasma obtained from the blood of healthy donors (5mL). Fibrin networks were developed from 1 mL of this labelled plasma (by adding 1U/mL bovine thrombin (Parke Davis) and 25mM  $\text{CaCl}_2$  (final concentration)) and allowed to incubate at 37°C for 30 min. The clots were incubated 37°C for 3 hours using an elliptical rotator with 3mL unlabelled whole blood  
10 or 3mL unlabelled plasma. The radioactivity of incubation media was measured using a gamma counter.

#### Results:

50 $\mu$ l plasma + <i>TT</i> (initial count)	58700cpm
50 $\mu$ l blood incubated with labelled clot	380cpm
50 $\mu$ l plasma incubated with labelled clot	450cpm

Crushed clot on spatulum	1026700cpm
--------------------------	------------

Conclusion: *ThromboTrace* was tightly bound to fibrin, washout of label into surrounding blood or plasma was less than 1 %.

5

### Example 11

Study of the binding of *ThromboTrace* to fibrin under perfusion.

Protocol: Fibrin network was developed in pre-etched glass tube from 1 mL purified fibrinogen solution (2.5mg/mL) by adding 1U/mL bovine thrombin (Parke Davis) and 25mM $\text{CaCl}_2$  (final concentration). The network was gently perfused with 1.5mL normal saline containing *ThromboTrace* (TT) (50 $\mu$ l). The radioactivity of expelled perfusate was measured using gamma counter. Clots were then crushed with a wooden spatulum washed in normal saline and counted using a gamma counter.

#### Results:

50 $\mu$ l NS+TT (initial count)	47500cpm
50 $\mu$ l expelled perfusate	3200cpm
Crushed clot on spatulum washed with NS	991200cpm

Conclusion: After the perfusion of normal saline containing *ThromboTrace* through fibrin network only 7% of initial radioactivity was detected in the perfusate while crushed clot retained considerable radioactivity. This observation indicates a high affinity of *ThromboTrace* to purified fibrin. This experiment was repeated with plasma clots and similar results were observed.

### Example 12

Study of the specific binding of *ThromboTrace* to fibrin by autoradiography.

Protocol: Fibrin network was developed in pre-etched glass tube from 1 mL purified fibrinogen solution (2.5mg/mL) by adding 1U/mL bovine thrombin (Parke Davis) and 25mM $\text{CaCl}_2$  (final concentration). The network was gently perfused with 1.5mL normal saline containing *ThromboTrace* (TT) (50 $\mu$ l). The tube was autoradiographed with a gamma camera (Fig.1).

**Conclusion:** Clots layered with *ThromboTrace* and autoradiographed with a gamma camera revealed extensive binding of material within the first cm of perfusion of the clot.

### Example 13

5       **Study of the binding of *ThromboTrace* to fibrin in dynamic situation using an artificial circuit.**

**Protocol:** The artificial circuit is an adaptation of the Chandler Loop attempting to simulate the vascular environment of the body. This experimental set up allows the monitoring of the imaging of clots with the control of the parameters of flow and  
10       pressure.

      Fibrin network was developed from pooled human plasma obtained from healthy donors (by adding 1U/mL bovine thrombin (Parke Davis) and 25mM $\text{CaCl}_2$  (final concentration)) in a specially designed glass cell with a depressed well to house the clot. This design simulated an intramural clot with its surface exposed to the flowing  
15       medium. The clot was allowed to incubate at 37°C for 30 min. The cell was connected with silicone tubing to the circuit comprised of a peristaltic pump and manometer (Fig.2).

      The circuit was filled with 50mL normal saline containing 10% pooled human plasma and 1 mL *TT* (2mCi/mL). The cell with clot was tilted so that the circulation mixture  
20       was not allowed to accumulate on top of the clot and was only trickling along the surface of the clot. A separate hanging clot was developed within a loop of copper wire and introduced into the circuit so that the total surface of the clot was exposed to the circulating media. Images were dynamically recorded using a gamma camera. Data acquisition time was 30 min. After that the circulation mixture was removed, the circuit  
25       was washed with normal saline and the image of the clot was acquired for another 10 minutes.

#### **Results:**

      Fig. 3 shows the image of the clot after 30 min of dynamic labelling with *ThromboTrace* and after the silicone tubes and the clot were washed with normal saline.  
30       The bright spot in the circuit above the clot is that of the hanging clot formed within a small copper wire loop and simulating small clots found in the body.

Conclusion: It is obvious that both clots have been labelled with *ThromboTrace* and show up clearly.

#### Example 14

Study of the binding of *ThromboTrace* to fibrin using Transmission Electron  
5 Microscopy.

Protocol: Fibrin networks were developed from purified fibrinogen solution or plasma containing *TT* (by adding 1U/mL bovine thrombin (Parke Davis) and 10mM  $\text{CaCl}_2$  (final concentration)). They were primary fixed in 2.5% glutaraldehyde and postfixed in 2% osmium tetroxide. They were then stained en block with 2% uranyl  
10 acetate, washed in distilled water, dehydrated and embedded in Spurr resin. The samples were cut on a Reichert-Jung Ultracut, stained with Reinold's lead citrate stain and micrographs taken on Philips CM 10 TEM. Negative stained fibrin clots with *TT* were prepared on carbon grids preliminarily treated with glow discharge. The grids were washed in distilled water, dried and stained in 1% sodium phosphotungstate. They  
15 were then briefly washed and dried. Subsequently, micrographs were taken on a Philips 301 TEM.

Results and Conclusions: Fig.4 shows transmission electron micrography which reveals particles adhering to fibrin along fibres and specifically bound to dark bands of negatively stained fibrin thus denoting a specific binding site for particles.

20

#### Example 15

Study of the binding of *ThromboTrace* to fibrin monolayer using microwell technique.

Protocol: 96 well PVC plates were coated with one of the following: PBS, solution of bovine serum albumin (BSA)/PBS, D-dimer, soluble fibrin, fibrin. The  
25 concentration of proteins directly coating wells was 10 $\mu\text{g/mL}$ . If proteins were raised from the surface by using 4D2/182 monoclonal antibody specific to fibrin(ogen) (AGEN) (10 $\mu\text{g/mL}$ ), the concentration of proteins was 100 $\mu\text{g/mL}$ . Wells were washed 3 times with PBS/BSA buffer in between application of proteins or *TT*. Plates were incubated with *TT* at 37°C for 30 min. Plates were washed with PBS/BSA buffer and  
30 each well was counted using a gamma-counter.

Results:

Protein	% binding of <i>TT</i> compared to BSA-treated wells
Fibrinogen	38
Plasma + thrombin	177
D-dimer	0
Fibrin	179
Soluble Fibrin	189

**Conclusions:** *ThromboTrace* binds with high affinity to fibrin monolayer and soluble fibrin. This affinity is not influenced by albumin or fibrinogen. In separate experiments it has been demonstrated that increasing the amount of fibrinogen does not inhibit the binding of *TT* to fibrin. *ThromboTrace* does not bind to D-dimer or albumin.

5

#### Example 16

Study of the effect of surfactant (C<sub>16</sub>E0<sub>6</sub>) on the binding of *ThromboTrace* to fibrin.

**Protocol:** The studies have been performed using multiwell technique (Example 15). *TT* solution was prepared with different concentration of surfactant.

10

#### Results:

Concentration of the surfactant (%)	% binding of <i>TT</i> compared to 0 concentration of the surfactant
0.0015	140
0.003	258
0.00625	194
0.0125	168



23

0.025	133
0.05	139

**Conclusions:** Binding is enhanced with C<sub>16</sub>E<sub>06</sub>. Maximal binding is observed at the concentration of 0.003%.

5 The *in vivo* experiments described below were carried out on rabbits and a cat adhering to standard anaesthetising procedure according to the guidelines provided by the Australian National Health and Medical Research Council. All animals were injected with 1 mL *ThromboTrace* (2mCi/mL). Acquisition time for all images was 60 min.

10

#### Example 17

##### Imaging of clots in rabbit ear vein.

Two needlestick injuries with 20G needles were made in the right ear and one in the contralateral ear. *ThromboTrace* was injected into the ear vein of the right ear. As shown in Figure 5 both right ear and contralateral ear injuries have been labelled with  
15 *ThromboTrace*.

#### Example 18

##### Clot caused by intramuscular injection of anaesthetic.

Fig 6 shows a clot caused by intramuscular injection of anaesthetic in the left thigh. *ThromboTrace* was injected into the ear vein. This photograph is representative of  
20 imaging done both one hour after the injection and 24 hours post injury.

#### Example 19

##### Imaging of clot in cat femoral vein.

Needlestick injury was made in the femoral vein of a cat. *ThromboTrace* was introduced near the site of injury after one hour. The labelled clot is clearly visible in  
25 the Figure 7. Further along the site of injury is a set of four bright spots. It is suspected that these were clots formed by previous injury to the animal. It was not possible to confirm this finding.

**Example 20****Imaging of clot in rabbit pulmonary vasculature.**

Human blood clot was developed around a cotton thread in a polyurethane tube (diameter=3mm) with 1U/mL thrombin and 10mM $\text{CaCl}_2$ . After washing with normal saline a 1mm length clot was introduced through a 16 or 18G catheter into the jugular vein of a rabbit. After 10 min *ThromboTrace* was introduced through the catheter or through the central ear artery. The results are shown in Figure 8. Classic wedging denoting depletion of vascular supply was seen in all cases.

**Example 21****10      Autoradiography of rabbit lungs with pulmonary emboli.**

Lungs were removed from the rib cage and autoradiographed to match images of clots obtained *in vivo*. The introduced human clot with cotton thread was then identified in the lung on post mortem and confirmed by two other witnesses and agreed that images obtained during the *in vivo* acquisition were in fact the introduced human blood clot.

15      The results are shown in Figure 9.

**Example 22****Specificity of particles for equine fibrin.**

*In vitro* experiments using plasma clots developed from equine blood also shows similar specificity of particles for equine fibrin. Studies utilised methods as described in

20      Example 9. No significant difference was found between the binding characteristics of *TT* to horse fibrin and human fibrin.

**Example 23****Binding to tumours.**

Fig 10. shows the labelling of a small tumour on the ear of a rabbit. The

25      experiment was performed as described in Example 7. Studies show that tumours on rabbit ear can be labelled using *ThromboTrace*, and the carbon nano-encapsulates may thus be used in labelling tumours and cancers which have a fibrin sheath.

**Example 24****Bone Scan.**

Bone breakages are followed by a deposition of fibrin. In particular, hairline fractures are often difficult to distinguish and diagnose. The propensity of the carbon nano-encapsulates to bind to fibrin provides another application as a label for bone fractures and therefore a frontline bone scan.

5

#### Example 25

##### Binding to soluble fibrin.

High affinity binding of *ThromboTrace* to soluble fibrin can be used as a basis for a diagnostic test for soluble fibrin which is reportedly a marker for cardiovascular disease and cancer among other diseases, eg Bredbacka *et al.* (1994 a & 1994 b), Ginsberg *et al.* (1995), Ginsberg *et al.* (1996), Iversen *et al.* (1995), Nakagawa *et al.* (1994), Shaukat *et al.* (1995). Experimental data set out in Example 15 demonstrates the binding of *ThromboTrace* to soluble fibrin.

#### Example 26

##### Diagnostic kit for soluble fibrin.

15 Soluble fibrin bound particles are isolated and a suitable monoclonal antibody specific for soluble fibrin is attached to the soluble fibrin. An ELISA assay is then developed to specifically detect the quantity of the soluble fibrin in plasma.

Colloidal gold may be incorporated into these particles in order to strengthen the integrity of particles and increase the mass, thus increasing the sensitivity of the immunogold assay.

20 The affinity of the particles to soluble fibrin can then be used to detect the quantity of soluble fibrin again by utilising the immunogold type technique. Alternatively, it may be possible that the aggregation of particles caused when they bind to soluble fibrin may be used as the method of detection as these particles may exhibit a subtle colour change because of the aggregation or difference in light scattering.

#### Example 27

##### Targeting Specific Drugs.

30 A novel use for the nano-particles is the coupling of anticoagulant drugs or clot specific drugs to the carbon shell. This can be established by the functionalisation of the carbon by amination or hydroxylation. These procedures will be accomplished using a radiofrequency plasma field. Once functionalised these particles will then be coupled to

drug molecules using commercially available kits. In fact one anticoagulant already available is biotinylated heparin which can then be coupled to the carbon shell.

Coupling to a drug is followed by stripwell tests to ensure binding to fibrin is not disrupted. These tests also show the effectiveness of these drugs when bound to the carbon. These coupled particles are then injected into an animal. The first injections are with radioactive technitium based particles which enables detection and further describes the kinetics of dissolution of the clots. If particles are coupled with tissue plasminogen activator for example, this indicates a rapid dissolution of the clot. If the particles are heparin coupled these experiments will provide ample evidence that the heparin is in fact delivered to the site it is required. This is one major disadvantage of heparin usage at the moment.

#### Industrial Applicability

It should be clear that the methods of detection of this invention will find wide use in the medical and veterinary fields.

The foregoing describes only some embodiments of the present invention and modifications obvious to those skilled in the art can be made thereto without departing from the scope of the invention.

#### References

- Bredbacka *et al.* Soluble fibrin: a predictor for the development and outcome of multiple organ failure. *Am J Hematol* 46:289-294, 1994 (a)
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- Ginsberg *et al.* Evaluation of a soluble fibrin assay in patients with suspected deep vein thrombosis. *Thromb Haemost* 75(3):833-836, 1995
- Ginsberg *et al.* Evaluation of a soluble fibrin assay in patients with suspected pulmonary embolism. *Thromb Haemost* 75(4):551-554, 1996
- Iversen *et al.* Soluble fibrin in plasma before and after surgery for benign and malignant colorectal disease. *Thromb Res* 79:471-481, 1995
- Nakagawa *et al.* Plasma levels of soluble fibrin in patients with malignancy-associated disseminated intravascular coagulation. *Blood Coagul Fibrinolysis* 5:725-730, 1994

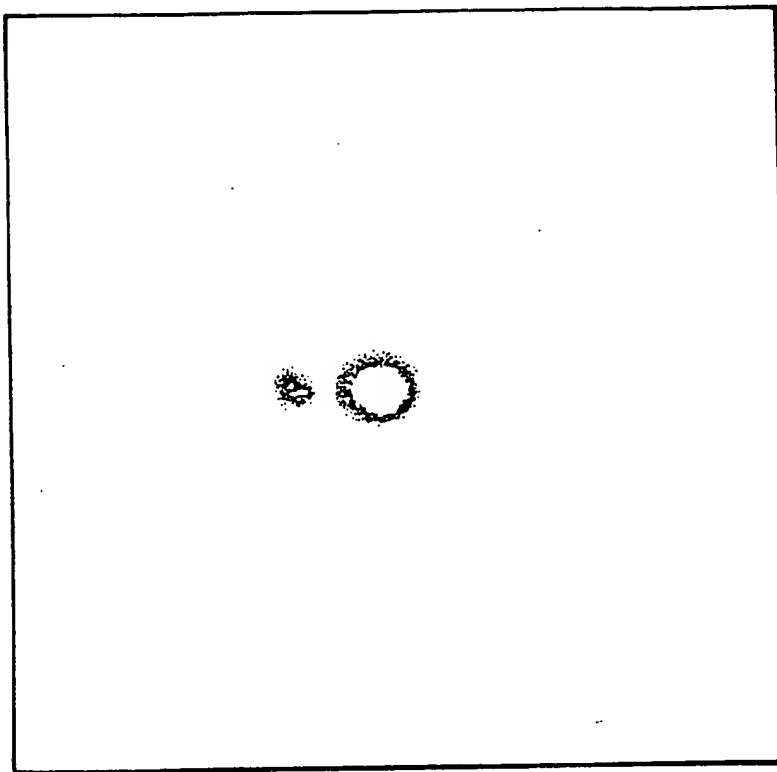
Shaukat et al. Soluble fibrin: a marker of the development of thrombosis in coronary heart disease. *Thromb Haemost* 73(6):1141 (922), 1995

## CLAIMS

1. A reagent for use in labelling macromolecules, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon.
2. A method for labelling a macromolecule with a detectable marker, which method  
5 comprises contacting said macromolecule with reagent for use in labelling macromolecules, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon whereby the reagent binds to the macromolecule to form a reagent:macromolecule complex.
3. The method of claim 2 further comprising detecting the reagent macromolecule  
10 complex by detecting the detectable marker.
4. A method for labelling a biological macromolecule in a patient, which method comprises administering to the patient, an effective amount of reagent for use in labelling macromolecules, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon.
- 15 5. A method for detecting the presence of a macromolecule in a source, which method comprises contacting the source with a reagent for use in labelling macromolecules, comprising a detectable marker encapsulated by a nano-encapsulate entity comprising carbon, said reagent being capable of forming a complex with said macromolecule if present, and detecting for the presence or otherwise of a  
20 macromolecule:reagent complex wherein presence of a macromolecule:reagent complex is indicative that the macromolecule is present in the source and absence of macromolecule:reagent complex is indicative that the macromolecule is not present in the source.
6. The reagent of claim 1 in the form of an injectable composition containing a  
25 suitable carrier.
7. The reagent of claim 6 wherein the carrier is saline and the detectable label is present in an amount of less than 100ng
8. The reagent of claim 1 wherein the detectable marker is a marker detectable by radiochemical techniques, or by magnetic resonance imaging or by visual methods.
- 30 9. The reagent of claim 1 wherein the detectable marker is a radionuclide selected from the group consisting of  $^{111}\text{In}$  with a half-life of 2.8 days,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{99\text{m}}\text{Tc}$ .
10. The reagent of claim 1 wherein the detectable marker is a visually detectable marker.

11. The reagent according to claim 10, wherein the detectable marker is a visually detectable colloid.
12. The reagent of claim 11 wherein the detectable marker is colloidal gold.
13. The reagent of claim 1 wherein the detectable marker is a magnetic resonance  
5 imaging marker.
14. The reagent of claim 13 wherein the detectable marker is Gd.
15. The method of any one of claims 2 to 5 wherein the reagent is in the form of an injectable composition containing a suitable carrier.
16. The method of any one of claims 2 to 5 wherein the detectable marker is a marker  
10 detectable by radiochemical techniques, or by magnetic resonance imaging or by visual methods.
17. The method of any one of claims 2 to 5 wherein the detectable marker is a radionuclide selected from the group consisting of  $^{111}\text{In}$  with a half-life of 2.8 days,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ , and  $^{99\text{m}}\text{Tc}$ .
18. The method of any one of claims 2 to 5 wherein the detectable marker is a  
15 magnetic resonance imaging marker.
19. The method of any one of claims 2 to 5 wherein the detectable marker is a magnetic resonance imaging marker and is Gd.
20. The method of any one of claims 2 to 5 wherein the macromolecule is a protein.
21. The method of any one of claims 2 to 5 wherein the macromolecule is fibrin.  
20
22. The method of any one of claims 2 to 5 wherein the detectable marker is a visually detectable marker.
23. The method of any one of claims 2 to 5, wherein the detectable marker is a visually detectable colloid.
24. The method of any one of claims 2 to 5 wherein the detectable marker is colloidal  
25 gold.

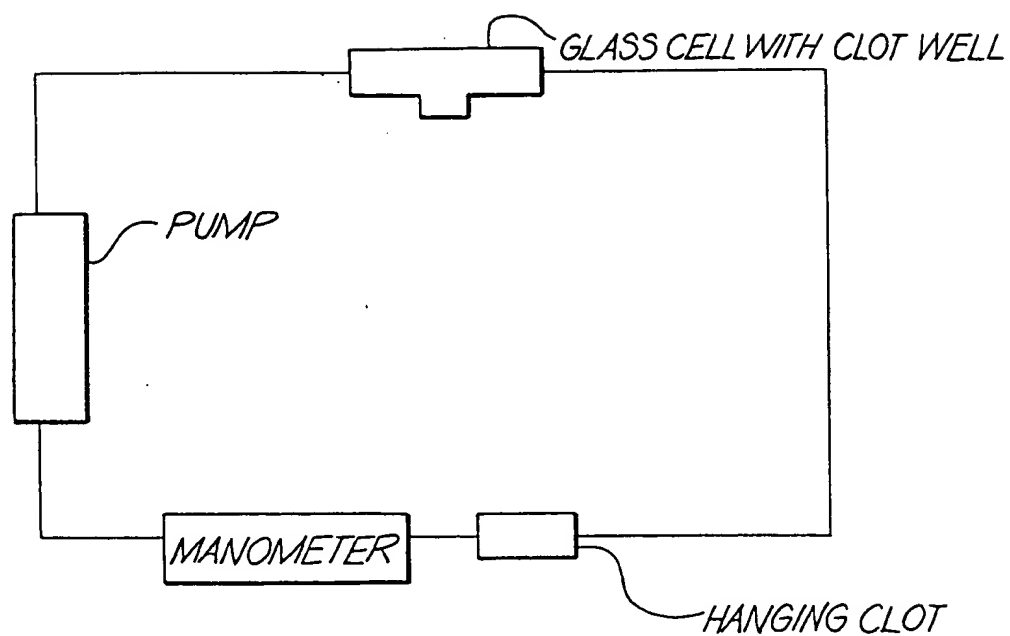
*SPECIFIC BINDING OF THROMBO TRACE TO  
SURFACE OF A CLOT AS SEEN BY AUTORADIOGRAPHY.*



*FIG. 1*

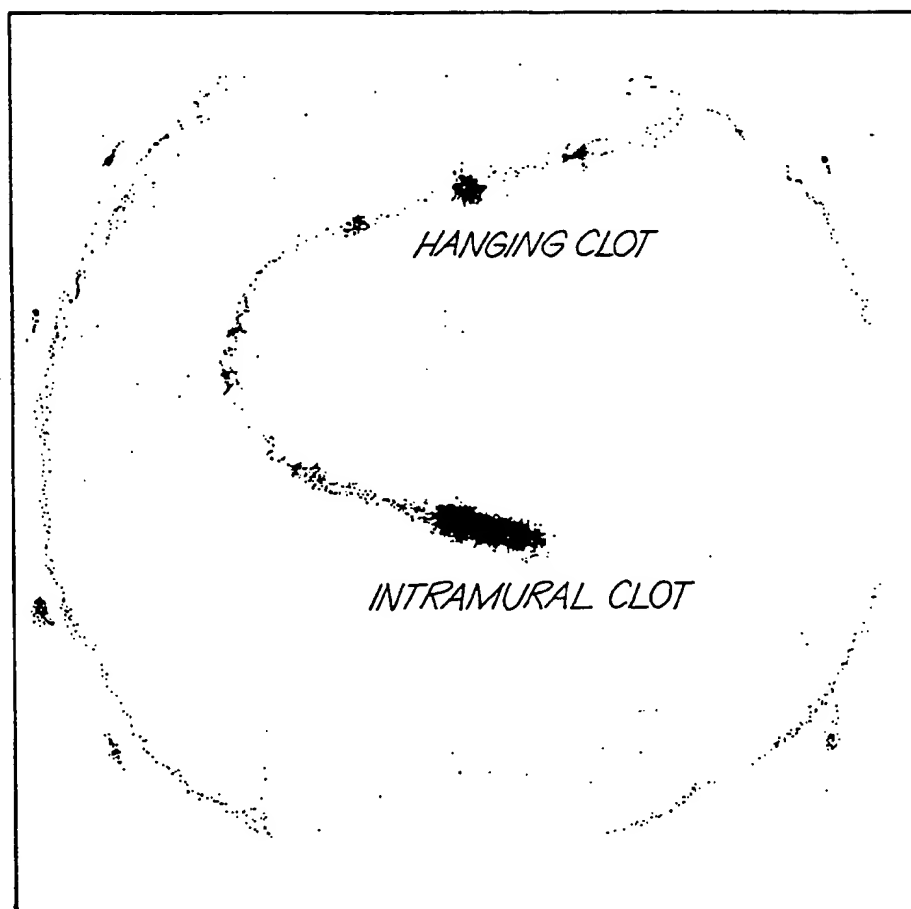


2/11

*ARTIFICIAL CIRCUIT**FIG. 2*

3/11

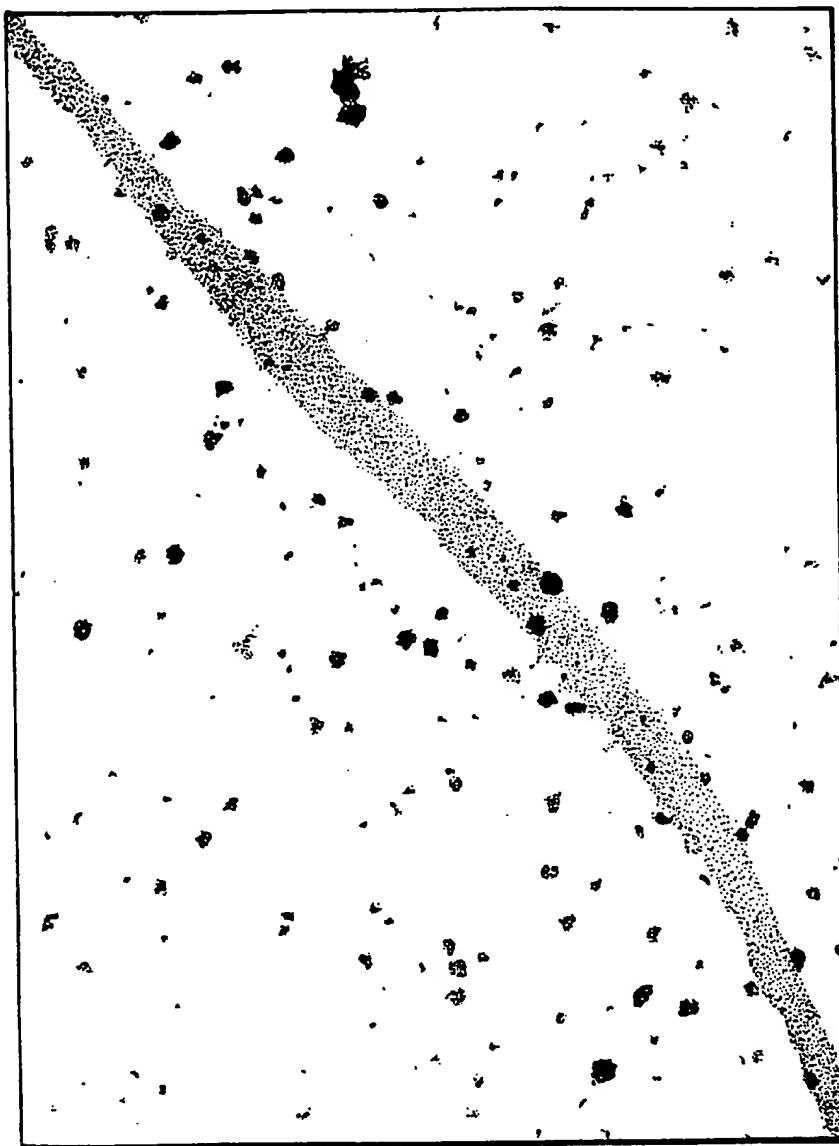
*THE ACCUMULATION OF RADIOACTIVITY IN THE  
INTRAMURAL CLOT IN AN ARTIFICIAL CIRCUIT.*



*FIG. 3*

4/11

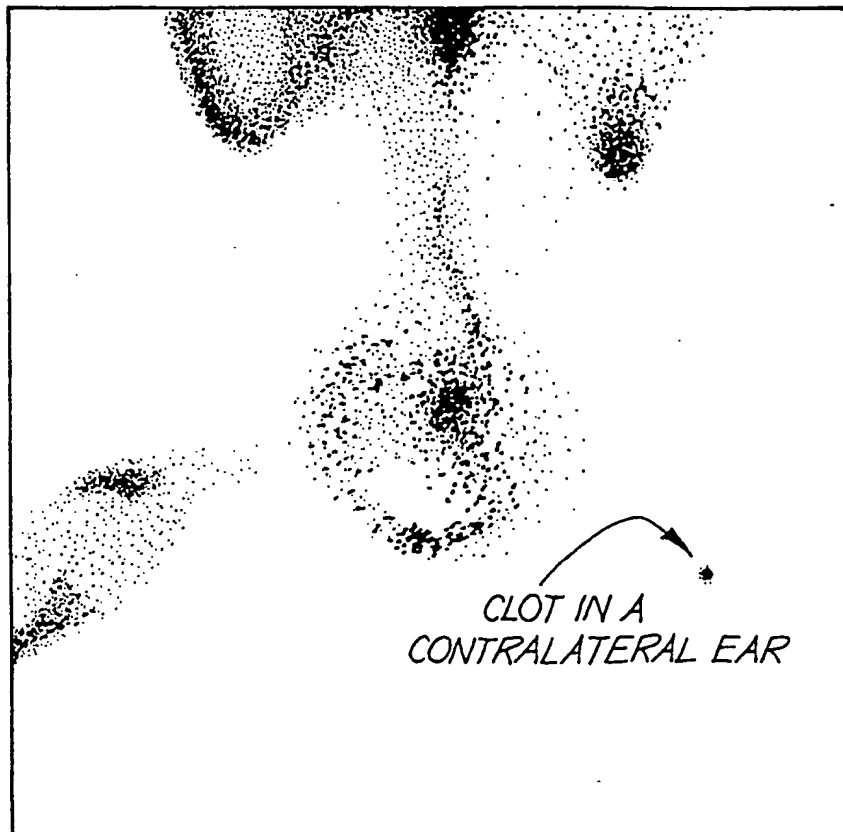
*TRANSMISSION ELECTRON MICROGRAPHY OF NEGATIVELY  
STAINED FIBRIN WITH THROMBO TRACE.*



*FIG. 4*

5/11

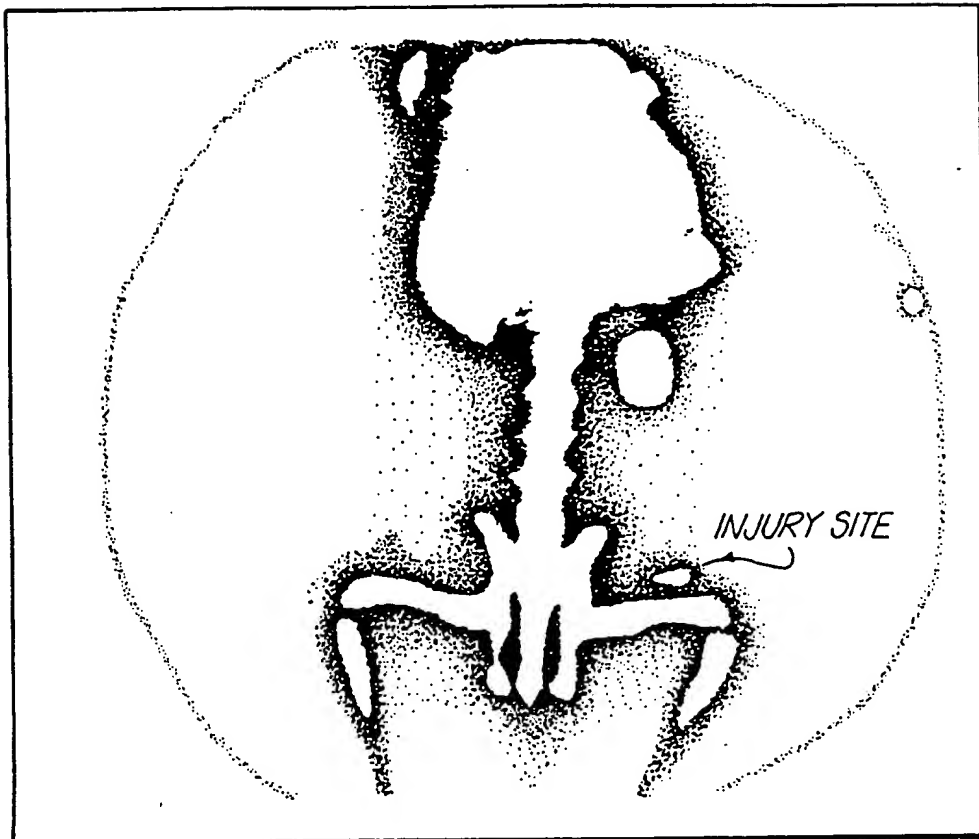
*IMAGING OF CLOTS IN RABBIT EAR VEIN.*



*FIG. 5*

6/11

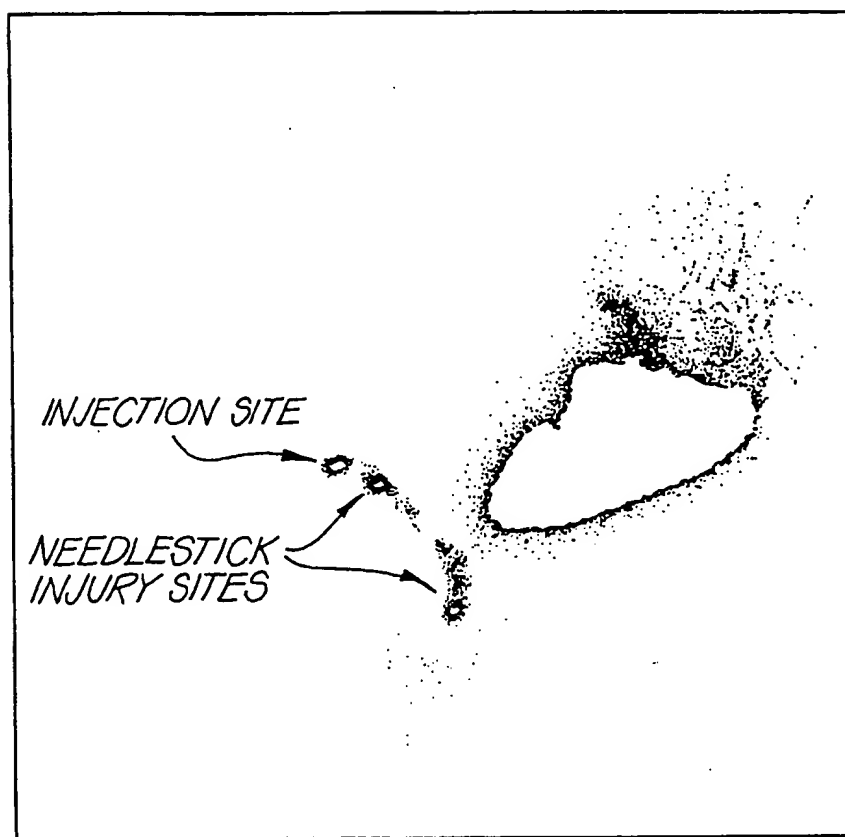
*IMAGING OF A CLOT IN RABBIT CAUSED BY  
INTRAMUSCULAR INJECTION*



*FIG. 6*

7/11

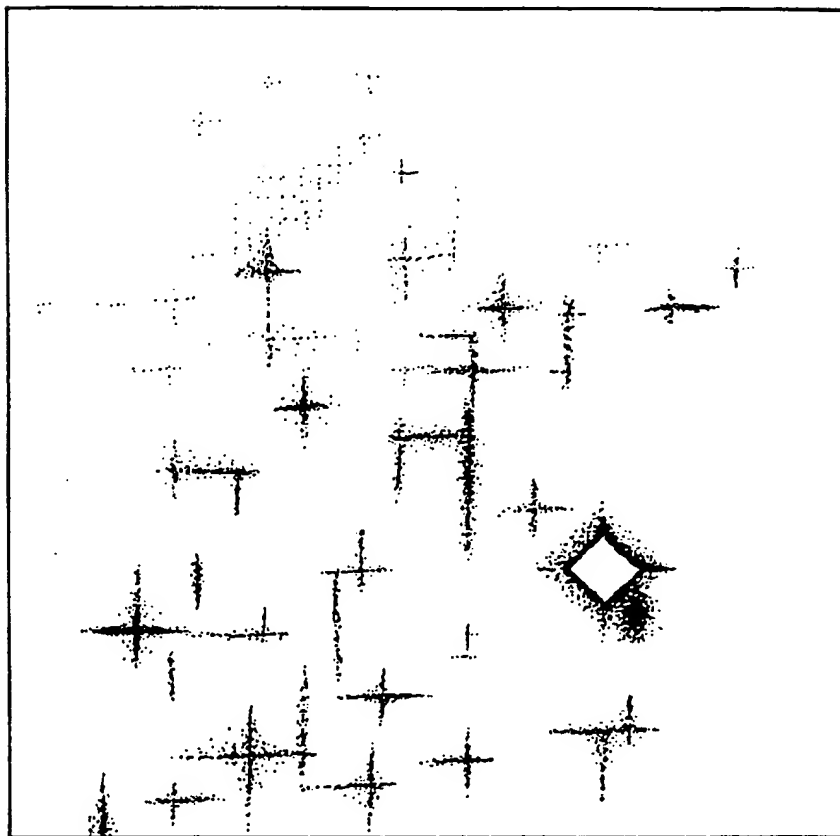
*IMAGING OF A CLOT IN THE CAT FEMORAL VEIN.*



*FIG. 7*

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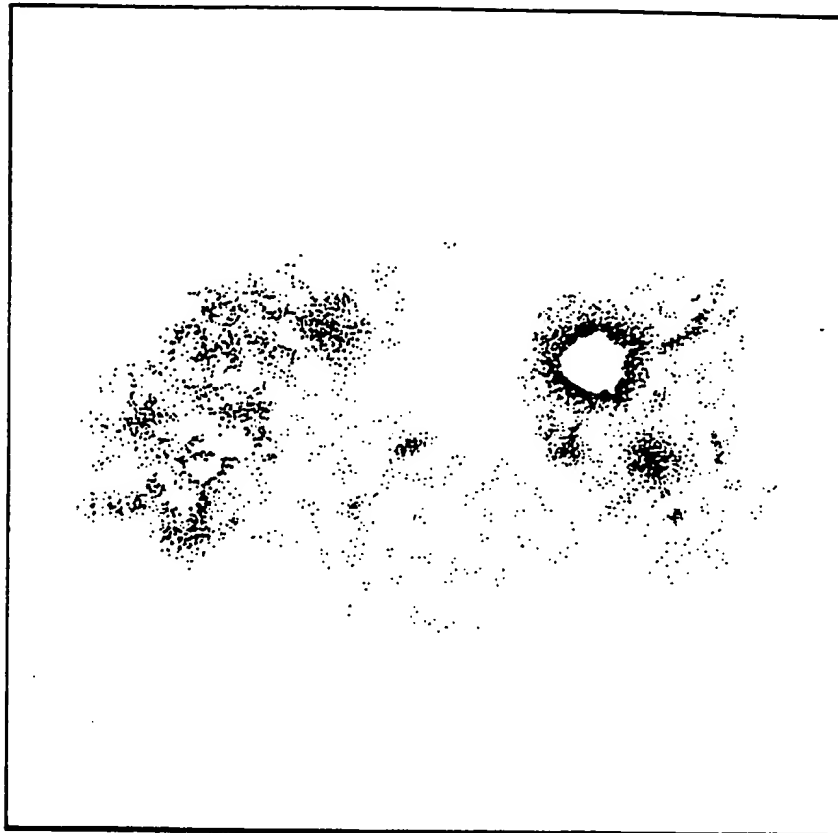
*IMAGING OF A CLOT IN RABBIT PULMINARY  
VASCULATURE.*



*FIG. 8*

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*AUTORADIOGRAPHY OF RABBIT LUNGS WITH  
PULMINARY EMBOLI.*

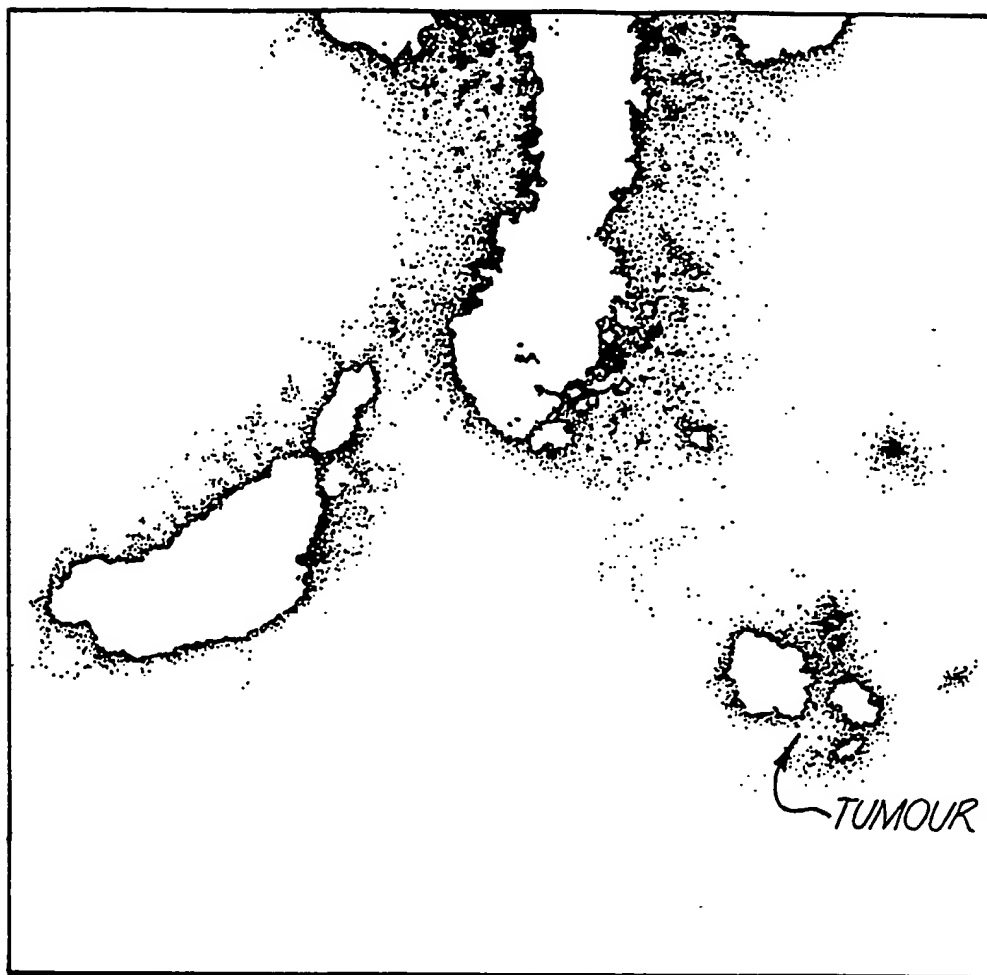


*FIG. 9*



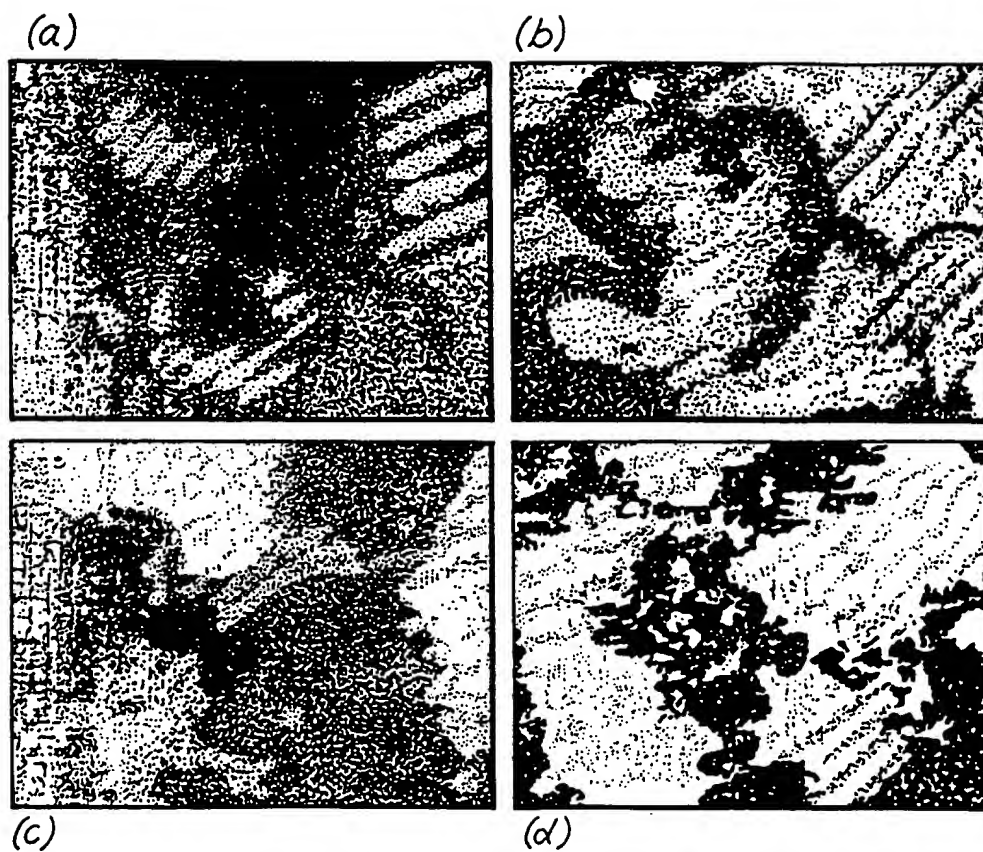
10/11

*LABELLING OF A SMALL TUMOUR ON THE EAR OF  
A RABBIT*



*FIG. 10*

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*FIG. 11*

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/AU 97/00467**

## A. CLASSIFICATION OF SUBJECT MATTER

Int Cl<sup>6</sup>: **A61K 51/02, 51/12**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int Cl<sup>6</sup> **A61K 51/02, 51/12; Int Cl<sup>5</sup> A61K 49/00, 49/02**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

FILE CA: (CARBON OR GRAPHIT?) AND (? ENCAPSUL?) AND (? LABEL?)

FILE CA: TECHNIGAS

DERWENT FILE WPAT: (CARBON OR GRAPHIT:) AND (A61K 49/00, 49/02, 51/02 OR 51/12)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/15768 A1 (NYCOMED SALUTAR INC) 19 August 1993 page 6 line 8-page 7 line 6, page 18 line 4-page 19 line 22	1-20, 22, 23
X	WO 93/03771 A1 (MALLINCKDROOT MEDICAL, INC) 4 March 1993 page 8 line 11-page 10 line 6	1, 6, 8, 13, 14
X	AU 48324/85 (589578) B (TETLEY MANUFACTURING PTY LTD) 10 April 1986 see whole document	1, 8, 9

☒ Further documents are listed in the continuation of Box C

☒ See patent family annex

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

10 September 1997

Date of mailing of the international search report

**15 SEP 1997**

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 97/00467

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU 31778/95 A (ALLRAD NO. 28 PTY LTD et al.) 4 April 1995 page 1A lines 5-11, page 5 lines 3-7	1, 6, 7, 8, 9
X	Appl. Radiat. Isot. (1995) Volume 46, No. 3 pages 157-165 T Nozaki et al., "Production of Fine Aerosols Labelled with Various Radionuclides by Sublimation from a Graphite Boat, and Their Properties and Tracer Use" see whole document	1, 6, 7, 8, 9

### Information on patent family members

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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